THE CULTURE OF *AMŒBA PROTEUS* IN A KNOWN SALT SOLUTION.

D. L. HOPKINS[1] AND PERCY L. JOHNSON.

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Introduction.

Many methods[3] have been described for the culture of Amœba proteus; but in them very little attention has been directed to the salt composition of the media used. The method adhered to in this laboratory for several years is described by Edwards ('23). It consists of adding a few short pieces of timothy hay to spring or distilled water in finger bowls with subsequent inoculation with amœbæ and *Chilomonas*. Hopkins ('26), however, has shown that for certain types of physiological work this method is not satisfactory since the salt and H-ion concentrations do not remain constant. To obviate these difficulties he raised amœbæ in a modification of Ringer solution supplemented by a daily feeding schedule. Later he used another modification of Ringer solution which contained only the chlorides of calcium, sodium and potassium with a phosphate buffer, pH 6.6 (Hopkins, '28). In this solution it was found that the amœbæ could be raised only after a considerable period of adaptation. Amœbæ from a culture of any other composition placed into this solution, disappeared.

Aside from the adaptation process there was still another difficulty of equally as great importance. Hay contains considerable salt so that when it is added to cultures the inorganic compositions are altered in an unknown way. Roberts ('26) presents a salt analysis of timothy hay which will suffice to illustrate the salt composition (Table I.). The salt content of timothy hay is not always constant, varying from six to eight

[1] National Research Fellow.

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[3] Dawson ('28) gives a thorough review of this literature.

per cent. ash, depending upon the environmental conditions, *e.g.*, climate, altitude, etc.

TABLE I.

Salts	Per Cent. Dry Weight.
Total ash	7.70
Silica free	4.00
P_2O_5	0.4712
CaO	0.3942
Fe_2O_3	0.0393
Na_2O	0.0023
K_2O	2.375
Cl	0.239

It appears from this analysis of the inorganic composition that a considerable amount of physiologically active substances is introduced into amœbæ cultures with the hay, thus causing alterations in the composition of the culture media. An attempt was made, therefore, in the experiments described in the following pages, first, to remove the salts from the hay and then to adjust the salt composition favorably for the growth of amœbæ and yet to control the H-ion concentration.

METHODS AND RESULTS.

Two methods were used to extract salts from timothy hay, *A* and *B*.

A. Since hay contains protein probably in the form of ampholytes, it is expected that it holds salts in combination which are not removed when extracted with distilled water. The procedure adopted by Loeb ('22) for extracting the salts from gelatin when the H-ion concentration has a value equal to the isoelectric point of the ampholyte, was applied to timothy hay. The method used follows: 75 gm. hay were ground in a meat chopper and extracted two times with 1.5 liters of distilled water. The hay was then added to 1.5 liters of distilled water and the mixture brought to a H-ion concentration of pH 8.0 by the addition of 60 cc. *M*/20 KOH, then the solution was filtered off. After washing with distilled water the hay extract had a H-ion concentration of pH 6.6. Following this, more distilled water was added and the H-ion concentration increased to pH 4.5 by the addition of 10 cc. *M*/5 HCl. The acid was then washed out until the filtrate had a H-ion con-

centration of pH 5.8. The hay was then dried at a temperature of 50°. After burning a sample of this hay it was found that only 1.1 per cent. of the total dry weight remained as ash.

B. In extracting the salts from the hay as described above, considerable organic substance was removed. In order to avoid this, another method was resorted to which afforded better results. This method of electro-dialysis was used by Livingston ('07) for separating electrolytes from non-electrolytes in manure extracts. The apparatus used is represented in Fig. 1. In this

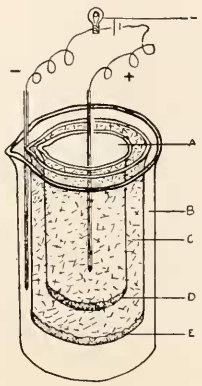

FIG. 1. Apparatus for dializing timothy hay. *A*, inner chamber containing distilled water into which is inserted the anode of the electric circuit; *B*, outer chamber containing distilled water into which is inserted the cathode; *C*, middle chamber containing finely ground hay; *D*, and *E*, collodion membranes (the collodion imbedded in bolting cloth for support); *L*, carbon lamp in the circuit to increase the resistance.

apparatus when the circuit [1] is closed the cations collect at the negative pole and the anions at the positive pole, leaving the undialyzable material behind. Frequently the acid and basic solutions formed at the two electrodes were replaced by distilled water. The dialysis was carried on for three days.[2] The hay was then dried in an oven at 50° C., after which a sample was tested and the ash content found to be 1.3 per cent. Since owing to the relative immobility of silicon ions it is very probable that 75 per cent. of the inorganic substances remaining in the

[1] A P. D. of 55 V. is best.

[2] Since it is difficult to prepare the collodion membranes, in the way used here, so that they will not leak, the use of diffusion shells is more practicable.

hay after dialysis consisted of silicon compounds and these are very inactive physiologically. This method of removing the salts appears to be very satisfactory.

This hay with very much reduced salt content was now used to culture amœbæ. We tried various mixtures containing different concentrations of the chlorides of calcium, sodium, potassium, and potassium phosphate buffer. After a number of attempts we found a mixture which on the first trial gave satisfactory results in all of fifteen cultures; the amœbæ on being placed in these cultures multiplied rapidly until at the end of two weeks each culture contained amœbæ in considerable numbers. The composition of this mixture is given in Table II.

TABLE II.

SOLUTION I.

$CaCl_2$	.02219 gm.	.0002 M.
NaCl	.23380 gm.	.004 M.
KOH	.0499 gm.	.00088 M.
KH_2PO_4	.34030 gm.	.0025 M.
Dialized hay extract	10 cc.	
H_2O	to 1,000 cc.	

This mixture was ordinarily prepared by making a solution of the first two mentioned salts thirty times as concentrated, then taking 33.3 cc. of this and adding to it 50 cc. of Clark and Lubb's phosphate buffer 6.6 (with KOH instead of NaOH); to this was added 2 gm. of the dialyzed hay, 10 cc. of the resulting dialyzed hay extract, a liberal amount of centrifuged *Chilomonas* or better *Colpidium* grown on the same media and distilled water to make 1,000 cc. The cultures made up in finger bowls, 100 cc. solution to a dish, were then inoculated with *Amœba proteus*.

The original success with this medium seems to have been accidental, since subsequent attempts to culture amœbæ in the medium were not universally successful. If fresh cultures were inoculated with amœbæ from the original fifteen cultures they were as successful as the originals, but if inoculated with amœbæ from spring water cultures uninterrupted growth and reproduction occurred in a relatively few cases. Therefore, it was concluded that in the inoculation of the original fifteen cultures, amœbæ were used, which accidentally happened to be in the right physiological condition, perhaps the culture from which

they were obtained had nearly the same composition as the new medium.

For inoculation with amœbæ from ordinary dilute spring water cultures, the buffer in the medium of Table II. appears to be too concentrated. Therefore, in order to insure success with amœbæ from dilute cultures, it is necessary to reduce the amount of buffer considerably, inoculate with amœbæ, then gradually increase the amount of buffer until sufficient buffer is present to keep the H-ion concentration constant. To do this the buffer content of the medium was reduced to 5 cc. standard buffer per 1,000 cc. medium and inoculated with amœbæ from ordinary spring water cultures. In the medium with this reduced buffer content, a high degree of success was obtained, in over 70 per cent. of the cultures, the amœbæ on being placed in it continued to grow and multiply such that within two weeks great numbers of amœbæ were present in the cultures. Then 10 cc. of standard buffer was added per 1,000 cc. culture. This addition did not noticeably affect the rate of growth and multiplication. In this manner by adding the buffer gradually healthy cultures of amœbæ, rapidly dividing by fission, could always be obtained, which contained sufficient buffer to hold the H-ion concentration constant, namely, 50 cc. standard buffer per 1,000 cc. culture.

Discussion.

In making up a 100 cc. culture with solution I., .2 gm. of hay is added. If this hay is undialyzed it contains approximately 8 per cent. salts or 0.016 gm. salts of an unknown nature. The weight of the known salts in 100 cc. of solution I. is 0.0647 gm. Therefore, if undialyzed hay is added, the unknown salt concentration of the culture is 19.9 per cent. of the total salts present. When .2 gm. of dialyzed hay, having from 1.0 to 1.4 per cent. inorganic material in it, is added in place of the undialyzed hay, there is introduced only from .002 to .0028 gm. unknown salts, *i.e.*, 2 to 3 per cent. As stated above since it is probable that the greater amount of the salts left in the dialyzed hay is silicon compounds, those that are left have little physiological effect.

Summary.

A medium, the inorganic composition of which is accurately known, was devised, in which *Amœba proteus* grows and multiplies rapidly. By adding potassium phosphate buffer gradually, so as to allow time for adaptation on the part of the amœbæ a concentration of buffer may be added which is sufficient to hold the H-ion concentration constant and yet not interfere with the growth and multiplication of the amœbæ.

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